1.0 Introduction

The murine local lymph node assay (traditional LLNA)[1] is an alternative skin sensitization test method that requires fewer animals and less time than currently accepted guinea pig (GP) tests (e.g., the guinea pig maximization test and the Buehler test). It also avoids animal discomfort that can occur in the GP tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the traditional LLNA as an acceptable alternative to GP tests for most testing situations.

The current LLNA applicability domain was one of several LLNA-related topics nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).[2] For this evaluation, the LLNA was assessed for its ability to correctly identify the sensitization potential of pesticide formulations and other products, metals, and substances tested in aqueous solutions.

The ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285*l*-3) charged ICCVAM with coordinating the technical evaluations of new, revised, and alternative test methods with regulatory applicability. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that an evaluation of the LLNA applicability domain should have a high priority for evaluation. A detailed timeline of this evaluation is provided in **Appendix A**. The updated ICCVAM-recommended LLNA test method protocol, a comparison of LLNA results for substances tested in two different mouse strains, and the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999, hereafter Addendum) are provided in **Appendices B**, **C**, and **D**, respectively.

The ICCVAM Immunotoxicity Working Group (IWG) was formed to work with NICEATM in evaluating the test methods. Dr. Silvia Casati was the European Centre for the Validation of Alternative Methods (ECVAM) liaison, and Dr. Hajime Kojima was the Japanese Center for the Validation of Alternative Methods (JaCVAM) liaison to the IWG.

To facilitate peer review of the LLNA applicability domain evaluation, the IWG and NICEATM, which administers ICCVAM and provides scientific and operational support for ICCVAM activities, prepared a comprehensive initial draft Addendum that provided information and data from validation studies and the scientific literature. A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815)[3] requested data and information on these test methods and nominations of individuals to serve on an international independent scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to this request, three individuals or organizations nominated members to the Panel (see **Section 4.0**).

In the initial draft Addendum, ICCVAM examined data derived from a database of over 500 substances (including pesticide formulations and other products) tested in the LLNA. In the original ICCVAM evaluation of the LLNA (ICCVAM 1999), the performance of the LLNA was compared to (1) results from GP tests and (2) information about sensitizers in humans (e.g., human

[1] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 2009a).

[2] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

[3] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

maximization test results, substances used in human repeat insult patch test, clinical case reports), where available. The initial draft Addendum updated the LLNA performance analyses for pesticide formulations and other products, metals, and substances tested in aqueous solutions when compared to human and GP results. On January 8, 2008, ICCVAM announced the availability of the initial draft Addendum to the public and a public Panel meeting to review the validation status of the LLNA applicability domain (and other LLNA-related activities) (73 FR 1360).[4] All of the information provided to the Panel, including the ICCVAM initial draft Addendum, draft test method recommendations, and all public comments received prior to the Panel meeting, were made publicly available via the NICEATM-ICCVAM website.[5]

The first Panel meeting was a public session held on March 4-6, 2008, to review the ICCVAM evaluation of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions and the completeness of the ICCVAM initial draft Addendum. The Panel evaluated (1) the extent to which the initial draft Addendum addressed established validation and acceptance criteria and (2) the extent to which the initial draft Addendum supported ICCVAM's draft proposed test method uses, recommended protocol, draft test method performance standards, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. The Panel recommended that NICEATM and ICCVAM solicit more data on pesticide formulations and other products and substances tested in aqueous solutions, before making recommendations about the usefulness of the LLNA for testing such substances. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix E**) on the NICEATM-ICCVAM website for public review and comment (announced in 73 FR 29136).[7]

ICCVAM provided SACATM with the updated draft Addendum and initial draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18-19, 2008, where public stakeholders were given another opportunity to comment.

NICEATM subsequently obtained a detailed test method protocol and data from an additional 140 substances and updated the initial draft Addendum to include this new information. The updated draft Addendum included an accuracy evaluation for the expanded database of over 600 substances (as compared with over 500 substances included in the January 2008 draft). Based on the analyses included in the updated draft Addendum, ICCVAM prepared updated draft test method recommendations for proposed test method uses and limitations, recommended protocol, test method performance standards, and future studies for the LLNA. ICCVAM released the updated draft documents to the public for comment on February 27, 2009, and announced a second meeting of the Panel (74 FR 8974).[8] The Panel reconvened on April 27-28, 2009, to again evaluate the LLNA applicability domain. The Panel also reviewed the completeness of the ICCVAM updated draft Addendum and the extent to which the information therein supported the ICCVAM updated draft test method recommendations. On June 1, 2009, ICCVAM posted the second report of the Panel's recommendations[9] (see **Appendix E**) on the NICEATM-ICCVAM website for public review and comment (announced in 74 FR 26242).[10]

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[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] Available at http://iccvam.niehs.nih.gov
[6] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[7] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf
[8] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf
[9] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf
[10] Announced in 74 *FR* 26242 http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-12360.pdf

ICCVAM provided SACATM with the revised draft Addendum, the second Panel report, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

After SACATM's meeting, ICCVAM and the IWG considered the SACATM comments, the Panel report, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and the Addendum provided in this report. As required by the ICCVAM Authorization Act, ICCVAM will make this test method evaluation report and the accompanying final addendum available to the public and to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. Agency responses will be made available to the public on the NICEATM-ICCVAM website as they are received.

2.0 ICCVAM Recommendations for the Updated Assessment of the Validity of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

ICCVAM has updated the original validation report of the LLNA (ICCVAM 1999) based on a comprehensive review of available data and information regarding the current validity of the LLNA for assessing the skin-sensitizing potential of pesticide formulations and other products, metal compounds, and substances in aqueous solutions. The information is based on a retrospective review of data derived from over 600 substances, including 104 pesticide formulations, tested in the LLNA. The current evaluation builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). The Addendum updates the LLNA performance analyses for pesticide formulations and other products, metal compounds, and substances in aqueous solutions when compared to (1) the results from GP tests and (2) information about sensitizers in humans (e.g., human maximization test results, substances used in human repeat insult patch test, clinical case reports), where available (see **Section 3.0** and **Appendix D**).

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

Pesticide Formulations: The current LLNA database contains test results on 104 pesticide formulations, 23 of which have comparative GP data. None have comparative human data. Ten out of the approximately 450 active ingredients registered with EPA were represented among these 23 formulations. Furthermore, approximately 40 different classes of pesticides are registered with EPA, of which these 10 active ingredients represent a small proportion (i.e., one insecticide, one microbioocide, six herbicides and two fungicides). Based on these 23 pesticide formulations, the concordance (accuracy) of the LLNA results compared to GP data is 57% (13/23), with an overprediction ("false positive") rate of 50% (10/20) and underprediction ("false negative") rate of 0% (0/3). Thus, there is a greater likelihood of obtaining a positive result in the LLNA (13/23; 57%) than in a GP test (3/23; 13%). All three formulations that were identified as positive in the GP tests were also identified as positive in the LLNA. Although human data are not available for these pesticide formulations to confirm their human sensitization potential, these data indicate that the LLNA is more likely to classify a pesticide formulation as a sensitizer than the GP tests. It should be noted that all 23 formulations were tested in the LLNA in the aqueous vehicle 1% Pluronic L92. Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects. If there is any primary testing or postmarketing reports of skin sensitization, they should be used for comparison with LLNA results.

The LLNA can be used for testing pesticide formulations unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of pesticide formulations may be a limitation of the LLNA.

Natural Complex Substances: The current LLNA database also contains data for 12 natural complex substances for which there are comparative LLNA and human data. Based on LLNA results for these natural complex substances, 75% (9/12) were sensitizers and 25% (3/12) were nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Based on this limited database, the concordance (accuracy) of the LLNA results compared to human sensitization data is 42% (5/12), with an overprediction ("false positive") rate of 75% (6/8) and underprediction ("false negative") rate of 25% (1/4). There are no comparative data from GP tests with these natural complex substances. Therefore, a comparison of the performance of the LLNA and the GP tests relative to the human outcome is not possible. Federal agencies should

assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing natural complex substances unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of natural complex substances may be a limitation of the LLNA.

Dyes: The current LLNA database contains data for six dyes, for which there are LLNA and GP data. Compared to GPMT outcomes, the LLNA concordance (accuracy) is 33% (2/6), the overprediction ("false positive") rate is 100% (1/1) and the underprediction ("false negative") rate is 60% (3/5). Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing dyes unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of dyes may be a limitation of the LLNA.

Metal Compounds: The current LLNA database contains test results on 48 studies involving 16 metal compounds representing 13 different metals (formulations containing metals are excluded from this analysis). All 16 metal compounds had comparative human data and eight had comparative GP data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. Because nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four, nickel compounds were excluded from the LLNA metals performance analysis.

For these remaining 14 metal compounds (13 metals), the LLNA concordance (accuracy) is 86% (12/14), the overprediction ("false positive") rate is 40% (2/5) and the underprediction ("false negative") rate is 0% (0/9), when compared to human results. The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative GP test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA concordance (accuracy) is 83% (5/6), the overprediction ("false positive") rate is 100% (1/1), and the underprediction ("false negative") rate is 0% (0/5), when compared to GP test results. When comparing the performance of the LLNA and the GP tests for the six metal compounds tested in all three species (i.e., mice, GPs, and humans) to human results, the LLNA concordance (accuracy) is 83% (5/6), the overprediction ("false positive") rate is 100% (1/1) and the underprediction ("false negative") rate is 0% (0/5). By comparison, the GP test concordance (accuracy) is 100% (6/6), the overprediction ("false positive") rate is 0% (0/1) and the underprediction ("false negative") rate is 0% (0/5) against the human. Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing metal compounds, with the exception of nickel, unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. Inconsistent results for nickel compounds obtained with the traditional LLNA suggest that the LLNA may not be suitable for testing substances containing nickel. Until the LLNA has been found to accurately identify ACD potential in substances containing nickel, further testing using a different test system is recommended when negative results are obtained for such substances.

Substances Tested in Aqueous Solutions: The current LLNA database contains test data on 44 studies that involved testing 25 substances in an aqueous solution. Pesticide formulations that were considered in the analysis discussed previously were also included in this evaluation, so this database

has the same limitations as discussed previously. The substances included in this evaluation contain at least 20% water. Most (23/25) of these substances were tested in the vehicle 1% Pluronic L92. Based on LLNA results for these substances 48% (12/25) were sensitizers and 52% (13/25) were nonsensitizers. However, based on GP results, only 20% (5/25) tested as sensitizers. Based on this limited database, the concordance (accuracy) of the LLNA compared to GP sensitization data is 56% (14/25), the overprediction ("false positive") rate is 48% (10/21) and the underprediction ("false negative") rate is 25% (1/4). Among the 11 substances for which LLNA and GP results were discordant, only one (i.e., neomycin sulfate) is negative in the LLNA and positive in the GP. These data suggest that the LLNA is more likely than the GP to classify a substance tested in an aqueous solution as a sensitizer. Human data are available for one substance that is discordant between the LLNA and the GP (i.e., neomycin sulfate). This substance is also discordant between the LLNA (i.e., negative) and the human (i.e., positive). Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle, to maintain the test substance in contact with the skin (e.g. 1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate exposure is achieved, as demonstrated by positive control results. It should be recognized that the potential for possible overclassification of aqueous substances may be a limitation of the LLNA.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concurred that the available data supported the ICCVAM updated draft test method recommendations for the LLNA with regard to testing pesticide formulations, dyes, natural complex substances, metal compounds and substances tested in aqueous solutions, in terms of the proposed test method usefulness and limitations.

On the basis of the available information, unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances, the Panel considered all of these test materials as candidates for testing in the LLNA, subject to the limitations outlined in the ICCVAM Test Method Recommendations.

2.2 ICCVAM Recommendations: Test Method Protocol

An updated version of the validated ICCVAM-recommended LLNA test method protocol has recently been developed (Appendix A of ICCVAM 2009a). This revised protocol is recommended for all future LLNA studies and includes the following key aspects:

- The high dose should be the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation. The measurement of ear swelling is a potentially valuable adjunct for identifying local irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.
- Inclusion of a concurrent vehicle control and positive control in each study is recommended.

Additionally, ICCVAM recommends that there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, the LLNA should be considered for use as a reduced LLNA test method protocol in which only the high dose is tested, thus further reducing animal use.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that updated information on various elements in the Addendum did not suggest the need for changes to recommendations for the development of a revised standard method. Whenever discretion is permitted, the Panel recommended the inclusion of a suitable (representative) positive control from the same category of materials to be tested (e.g., for testing pesticides, select one representative positive control pesticide).

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA:

- To more comprehensively evaluate the ability of the LLNA to be used for testing nickel compounds, additional data from LLNA studies on such compounds with comparative human and/or GP data are needed.
- Where available, solubility data should be provided in future studies so that thermodynamic activity can be computed and compared to maximum theoretical percutaneous penetration. This information should be considered when comparing the data from LLNA studies in lipophilic delivery systems compared to that in aqueous systems. Studies done in aqueous systems should use 1% Pluronic L92 as the vehicle in order to expand the existing database for that vehicle, unless adequate scientific rationale is provided for using another aqueous vehicle.
- Revalidation of the LLNA for new classes/types of test substances should be avoided unless there is a biologically based rationale. For new classes of test materials, an integrated assessment of available information should be conducted. This should include computer-assisted structure-activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding. Before any animal testing is conducted, consideration should be given to the necessity for a substance to be tested for skin sensitization potential.
- If any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concurred with ICCVAM's recommendations for future studies. The Panel also suggested that, before additional animal testing is conducted, consideration should be given to the necessity for the substance to be tested for skin sensitization potential.

2.4 ICCVAM Recommendations: Performance Standards

In conjunction with ECVAM and JaCVAM, ICCVAM has developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009a)[11] to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA.

[11] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm

3.0 Evaluation of the LLNA Applicability Domain

The following is a synopsis of the information in the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999) (**Appendix D**, hereafter, Addendum), which reviews the available data and information for the LLNA applicability domain. The Addendum describes the current validation status of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions, the scope of the substances tested, and standardized protocols used.

3.1 Test Method Description

The purpose of the LLNA test method is to identify potential skin sensitizers by quantifying lymphocyte proliferation in the draining auricular lymph nodes. The magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops after a topical induction exposure to a potential skin-sensitizing substance.

3.1.1 General Test Method Procedures

The LLNA measures lymphocyte proliferation after topical exposure to a potential skin-sensitizing substance. The test substance is administered topically on three consecutive days to the ears of mice at a concentration that provides maximum solubility of the test substance without causing systemic toxicity and/or excessive local irritation. Two days after the final application of the test substance, ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is administered via the tail vein. Five hours later the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the incorporation of radioactivity, which correlates with lymph node cell proliferation.

The incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine for each mouse is expressed in disintegrations per minute (dpm). The stimulation index (SI) is calculated as the ratio of the mean dpm/mouse for each treatment group against the mean dpm/mouse for the vehicle control group. The threshold for a positive response is an SI $\geq$ 3.

3.2 LLNA Applicability Domain Database

The information summarized in the Addendum is based on a retrospective review of LLNA data derived from a database of over 600 substances (including pesticide formulations and other products) tested in the LLNA and builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). To minimize duplication in this evaluation, metal formulations were not included in the analysis of pesticide formulations and other products, and metal compounds were restricted to those testing single substances. The reference database includes data for metal compounds from the original ICCVAM evaluation (**Appendix D,** Annex I), data published since that evaluation, and data submitted in response to a request in a FR notice (72 FR 27815)[12] requesting LLNA, GP, and/or human skin sensitization data and experience. An evaluation of the usefulness and limitations of the LLNA for testing pesticide formulations and other products, and substances tested in aqueous solutions was not included in the original ICCVAM validation (**Appendix D,** Annex I) because no data on these substances were available at that time. The reference database for these substances in the Addendum consists of data published since the original ICCVAM evaluation or submitted in response to the FR notice. **Table 3-1** provides information on the sources of the data and the rationale for the substances tested.

Among the LLNA studies for the pesticide formulations, 32% (29/89) used the BALB/c mouse strain rather than the CBA/J or CBA/Ca strains of mice, which are recommended in standardized LLNA

[12] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

protocols (ICCVAM 2009a; EPA 2003; OECD 2002). One additional submitted LLNA study (from Dr. Dori Germolec at the National Institute of Environmental Health Sciences [NIEHS]) also used the BALB/c strain. The comparative performance of the LLNA using these different mouse strains relative to the GP is detailed in **Appendix C**.

Table 3-1 Summary of Data Sources and Rationale for Substance Selection

Data Source	N	Substance Selection Rationale
AppTec Laboratory Services	48	Aqueous eluates from medical devices.
Dow AgroSciences	52	Pesticide formulations analyzed in the LLNA with associated GP data of various kinds.
Dupont	28	Pesticide formulations analyzed in the LLNA.
ECPA	39	Plant protection products (i.e., pesticides) were evaluated in the LLNA with a novel vehicle to assess its usefulness.
Basketter et al. (1994; 1996; 1999a; 2005)	16	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Lalko and Api (2006)	12	Original research that evaluated essential oils in the LLNA. RIFM and the authors submitted additional data.
Ryan et al. (2000)	2	Interlaboratory study to evaluate the accuracy of the LLNA to identify human sensitizers.
Ryan et al. (2002)	11	Original research with known water soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle in the LLNA.
E. Debruyne (Bayer Crop Science SA)	10	Original research on different pesticide types and formulations in the LLNA.
Kimber et al. (1991; 1995; 2003)	9	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Gerberick et al. (2005)[1]	6	Compiled from previously conducted LLNA studies (from published literature and unpublished sources) on substances of varying skin sensitization potential.
Bundesanstalt für Arbeitsschutz und Arbeitsmedizin	6	Original LLNA research on dye formulations.
H.W. Vohr (BGIA)	4	Original LLNA research with epoxy resin components as part of a validation effort for nonradioactive versions of the LLNA.
Basketter and Scholes (1992)[2]	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Gerberick et al. (1992)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
D. Germolec (NIEHS)	2	Substances were evaluated by NTP for skin sensitization potential in the LLNA.
Lea et al. (1999)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
M.J. Olson (GlaxoSmithKline)	2	Pharmaceutical substances tested in the LLNA.
Unilever (unpublished data)	2	Metal substances evaluated for skin sensitization potential in the LLNA.
Basketter and Kimber (2006)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Goodwin et al. (1981)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.

Continued

Table 3-1 Summary of Data Sources and Rationale for Substance Selection (Continued)

Data Source	N	Substance Selection Rationale
Griem et al. (2003)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Kligman (1966)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
J. Matheson (CPSC)	1	Published LLNA data submitted electronically to NICEATM, as a reference.
K. Skirda (CESIO - TNO Report V7217)	1	Data were provided by CESIO member companies for use in paper titled "Limitations of the LLNA as preferred test for skin sensitization: concerns about false positive and false negative test result".
Total	**262**	

Abbreviations:
BGIA = Berufsgenossenschaftliches Institut für Arbeitsschutz; CESIO = Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques; CPSC = U.S. Consumer Product Safety Commission; ECPA = European Crop Protection Association; ECVAM = European Centre for the Validation of Alternative Methods; GP = guinea pig; LLNA = murine local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIEHS = National Institute of Environmental Health Sciences; NTP = National Toxicology Program; RIFM = Research Institute for Fragrance Materials: TNO = Netherlands Organization for Applied Scientific Research.

[1] These data were evaluated by the ECVAM Scientific Advisory Committee in its evaluation of the LLNA limit dose procedure and were previously submitted to ICCVAM in 1998 for the original evaluation of the validation status of the LLNA (ICCVAM 1999; Gerberick et al. 2005).

[2] These LLNA studies used both male and female mice, but single experiments were limited to one sex.

3.3 Reference Test Method Data

The traditional LLNA data used for evaluation of the LLNA applicability domain include the results for all tested doses of each substance. In addition to calculated SI values for each of the tested doses, the vehicles tested and EC3 values (estimated concentration needed to produce an SI value of 3) for substances classified as sensitizers were provided in Gerberick et al. (2005). If EC3 values were not included in the data source, they were calculated, where possible, using either interpolation or extrapolation (Dearman et al. 2007).

The reference data for the GP tests (guinea pig maximization test [GPMT] or Buehler test) and human data (human maximization test, human patch test allergen, or other human data) were obtained from the scientific literature or from the data submitters. The complete database (by each source) is provided in Annex II, III, and IV of the Addendum (**Appendix D**).

3.4 Test Method Accuracy

Table 3-2 presents a summary of performance statistics for the LLNA for testing pesticide formulations, dyes, natural complex substances, metal compounds, and substances tested in aqueous solutions.

Table 3-2 Evaluation of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions

Comparison	n[1]	Accuracy		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]
Pesticide Formulations							
LLNA vs. GP[3]	23	57	13/23	50	10/20	0	0/3
Dyes							
LLNA vs. GP[3]	6	33	2/6	100	1/1	60	3/5
Natural Complex Substances							
LLNA vs. Human[4]	12	42	5/12	75	6/8	25	1/4
Metal Compounds							
LLNA vs. GP[4]	6	83	5/6	100	1/1	0	0/5
LLNA vs. Human[4]	14	86	12/14	40	2/5	0	0/9
Substances Tested in Aqueous Solutions							
LLNA vs. GP[3]	25	56	14/25	48	10/21	25	1/4

Abbreviations:
GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

False positive rate = the proportion of all negative substances that are falsely identified as positive

False negative rate = the proportion of all positive substances that are falsely identified as negative

[1] n = number of substances included in this analysis.

[2] The data on which the percentage calculation is based.

[3] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Pesticide Formulations: The current LLNA database contains data for 104 pesticide formulations. Among these formulations, 54% (56/104) were LLNA positive and 46% (48/104) were LLNA negative.

Seventy of the 104 pesticide formulations have LLNA and some type of associated GP reference data. A total of 89 LLNA studies were performed using these 70 formulations. LLNA studies were conducted with either CBA/Ca or CBA/J (61/89) and/or BALB/c (28/89) mouse strains. Six pesticide formulations were tested in multiple LLNA studies (25 studies total); 5/6 multiply tested pesticide formulations had LLNA results in agreement, and 1/6 pesticide formulations produced discordant results (i.e., three positive, two negative). The discordant data were for the pesticide formulation Oxyflourfen EC and were submitted to NICEATM by the European Crop Protection Association. In a five-laboratory study, SI values for the highest concentration tested (33%) ranged from 2.3 to 5.4. All lower concentrations tested showed no SI values ≥ 3.

All 70 pesticide formulations (89/89 studies) were tested in the LLNA in aqueous 1% Pluronic L92, a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA (Boverhof et al. 2008; Ryan et al. 2002).

Twenty-three pesticide formulations had associated GP data for the complete formulation, 46 pesticide formulations had GP data for one or more of the active ingredients included in the

complete formulation, and 14 pesticide formulations had GP data for a substance related to an active ingredient or for a related formulation.

For the 23 formulations for which there were GP data, the LLNA classified 52% (12/23) of the formulations as sensitizers while the GP tests classified only 13% (3/23) of the formulations as sensitizers. All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. Overall, the LLNA and the GP results were in agreement (accuracy) 57% (13/23) of the time (**Table 3-2**). The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the GP test, an overprediction (false positive) rate of 50% (10/20) (**Table 3-2**). Three of the LLNA studies for the 23 pesticide formulations were done with BALB/c mice. If these three studies are removed from the analysis, the LLNA and the GP results were in agreement 60% (12/20) of the time, and the overprediction was 47% (8/17). There were no instances of underprediction by the LLNA for these 23 pesticide formulations. Human data were not available for these pesticide formulations to confirm their sensitization potential in humans.

Dyes: The current LLNA database contains data for six dyes for which there are LLNA and GP data. Based on LLNA results for these six dyes, 50% (3/6) were sensitizers and 50% (3/6) were nonsensitizers. By comparison, based on GP results, 83% (5/6) were sensitizers and 17% (1/6) were nonsensitizers. The LLNA and the GP results were in agreement (accuracy) 33% of the time (**Table 3-2**). The overprediction (false positive rate) for the LLNA was 100% (1/1) and the underprediction (false negative rate) was 60% (3/5) (**Table 3-2**).

Natural Complex Substances: The current LLNA database also contains data for 12 natural complex substances (essential oils and absolutes) for which there are comparative LLNA and human data. Based on LLNA results for these substances, 75% (9/12) were sensitizers and 25% (3/12) nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Therefore, compared to human outcomes for these 12 substances, the LLNA was able to identify three out of four of the substances that were positive in human testing. However, an additional six substances that did not produce positive results in the human testing were positive in the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a false positive rate of 75% (6/8) and a false negative rate of 25% (1/4) (**Table 3-2**). There were no comparative data from GP tests with these substances. Therefore, a comparison of the performance of the LLNA and the GP tests relative to the human outcome was not possible.

Metal Compounds: The current LLNA database contains test results on 48 studies involving 16 metal compounds representing 13 different metals (formulations containing metals were excluded from this analysis). All 16 metal compounds had comparative human data and eight had comparative GP data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. Nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four. Two positive results occurred in aqueous vehicles, one positive result occurred in a nonaqueous vehicle, and the four negative results all occurred in nonaqueous vehicles. Because of these discordant results, a performance analysis for metals was also conducted with nickel compounds excluded.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12/14), a false positive rate of 40% (2/5) and a false negative rate of 0% (0/9), when compared to human results (**Table 3-2**). The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative GP test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA had an accuracy of 83% (5/6), a false positive rate of 100% (1/1), and a false negative rate of 0% (0/5) (**Table 3-2**), when compared to GP test results. When comparing the performance of the LLNA and the GP tests for the six metal compounds tested in all three species to human results, the LLNA had

an accuracy of 83% (5/6), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/5). By comparison, the GP tests had an accuracy of 100% (6/6), a false positive rate of 0% (0/1) and a false negative rate of 0% (0/5) relative to the human.

Substances Tested in Aqueous Solutions: The current LLNA database of substances tested in aqueous solutions includes results from 171 studies representing 139 substances; 91 (123 LLNA studies) of these substances are pesticide formulations and pure compounds, and 48 of these substances (48 LLNA studies) are aqueous eluates of medical devices. Because of differences in the protocols for sample preparation between the 91 pesticide formulations and pure compounds and the 48 medical device eluates, these groups were analyzed separately. Of the 91 pesticide formulations and pure compounds, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. LLNA studies were done with either CBA (66 studies) and/or BALB/c (28 studies) mouse strains. The mouse strain was unspecified for 29 studies. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water.

GP data were available for 25 (four sensitizers/21 nonsensitizers in the GP) substances tested in aqueous solutions. The outcomes of 11 substances were discordant between the LLNA and the GP tests. Ten of the 11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92; these were the same 10 substances previously discussed for the pesticide formulations analysis, and all were overpredicted by the LLNA with respect to the GP results (48% [10/21] false positive rate) (**Table 3-2**). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA with respect to the GP results (25% [1/4] false negative rate) (**Table 3-2**). Overall, the LLNA and the GP results were in agreement (accuracy) 56% (13/25) of the time (**Table 3-2**).

Human data were available for only four substances (three sensitizers/one nonsensitizer in humans) tested in aqueous solutions, while there were only two substances tested in aqueous solutions in the LLNA for which there was comparative GP and human data. Therefore, the database of substances tested in multiple test methods (i.e., LLNA, GP, and/or human) is too few to allow for a meaningful assessment of performance.

All 48 of the medical device eluates were negative in the LLNA. None of these eluates had associated GP or human data. These eluates were not analyzed to determine their constituents, or whether in fact any compound(s) were eluted from the medical device tested. Since the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined. Therefore, the results from these eluates were not included with those from the pesticide formulations and pure substances tested in aqueous solutions.

3.5 Animal Welfare Considerations: Reduction, Refinement, and Replacement

This comprehensive evaluation of the LLNA applicability domain should facilitate regulatory agency decisions on the acceptability of submitted LLNA studies for pesticide formulations and other products, metals, and substances tested in aqueous solutions. Following regulatory acceptance, use of the method by industry may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure. This can be expected to significantly reduce the number of animals required for ACD testing while continuing to support the protection of human health.

4.0 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the 12 different opportunities for public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA. The number of public comments received in response to each of the opportunities is also indicated. A total of 49 comments were submitted. Comments received in response to or related to the *Federal Register* notices are available on the NICEATM-ICCVAM website.[13] The following sections, delineated by *Federal Register* notice, briefly discuss the public comments received.

4.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of
    a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
    b. The reduced LLNA approach (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b)
    c. Nonradioactive LLNA methods
    d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
    e. The current applicability domain
2. Nominations of expert scientists to consider as members of a possible peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

In response to this FR notice, NICEATM received 17 comments. Six comments included additional data and information, while two others offered data and information upon request. Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the initial draft ICCVAM review documents that were provided to the Panel at the March 2008 meeting.

[13] Available at http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

Table 4-1 Opportunities for Public Comment

Opportunities for Public Comments	Date	# of Public Comments Received
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0
74 FR 8974: Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	February 27, 2009	1
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	April 28-29, 2009	2
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	0
74 FR 26242: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	June 1, 2009	1
SACATM Meeting, Hilton Arlington Hotel, Arlington, VA	June 25-26, 2009	0

1. A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

Two comments pertained to the LLNA applicability domain.

1. One commenter noted that the LLNA is the only method that can be used in the United Kingdom for assessment of skin sensitization potential for regulatory purposes and highlighted that in some areas of the chemical industry there is concern regarding the applicability of the LLNA for testing of preparations, mixtures and irritant substances. The commenter also noted that there is concern with regard to the view that the LLNA has not always provided results consistent with existing knowledge of the test substance or related test substances. The commenter indicated that since the LLNA offers significant scientific and animal welfare advantages over GP models for many product types, and, in the U.K., the LLNA is effectively the only available method for evaluation of skin sensitization potential for regulatory purposes, an assessment of the LLNA is welcomed.

ICCVAM initiated an assessment of the peer-reviewed literature and available data, and prepared a comprehensive background review document, to assess the LLNA applicability domain.

2. Another commenter indicated that available information should allow ICCVAM to make a rapid determination of the applicability and limitations of the LLNA for testing aqueous mixtures and metals, and, if not, then further validation efforts in this regard, should instead focus on *in vitro* methods.

In addition to *in vivo* refinement (less pain and distress) alternatives (such as the LLNA), ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

4.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols with regard to the traditional LLNA. In response to this FR notice, NICEATM received four comments, two of which suggested clarifications to the text. Another comment recommended that test substances chosen for testing in the various LLNA methods should be pure, with conclusive structures, and should not be mixtures. Most comments specifically addressed the LLNA performance standards, although one comment pertained to the LLNA in general.

1. One commenter supported the development of performance standards that expedite the validation of new protocols similar to previously validated methods but was disappointed that NICEATM-ICCVAM had chosen to develop performance standards for such a narrow scope of applicability (i.e., modifications of the standard LLNA that involve incorporation of nonradioactive methods of detecting lymphocyte proliferation). The commenter suggested that limited resources available to NICEATM-ICCVAM would be better spent on activities that would have greater impact on the reduction, refinement, or replacement of animal use, such as evaluating the use of human cell lines or *in vitro* skin models as a replacement for the LLNA.

ICCVAM considered the comment and concludes that the proposed modifications to the LLNA test method protocol and expanded applications have the potential to further reduce and refine animal use. ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

There were no comments that specifically addressed the LLNA applicability domain.

4.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the drafts for the January 2008 BRDs, ICCVAM test recommendations, test method protocols, and LLNA performance standards for an international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

Two written comments were relevant to the LLNA applicability domain.

1. One commenter indicated that the limited data prevented a conclusive recommendation for the use of the LLNA to predict the skin sensitization potential of mixtures, metals, and aqueous solutions. Thus, the commenter viewed that the approach to expand the applicability domain of the LLNA had not been successful, and recommended that further resources be directed towards the pursuit of *in vitro* methods.

ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

2. Another commenter indicated that the dataset used to evaluate mixtures was limited due to the lack of human data for comparison (i.e., only comparative GP data were available). The commenter questioned the likelihood that GP data is representative of the human response. Thus, they did not consider using GP data as reference data to be appropriate. In addition, the usefulness of the data was limited further by the fact that information on the ingredients was known for only one of the 15 mixtures and 11 were tested in the LLNA in an aqueous vehicle (noting that the usefulness and limitations of the LLNA for testing substances in aqueous solutions was also being evaluated).

    - As indicated in the January 2008 ICCVAM draft recommendations the limitations with the database indicated that more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made.

The commenter further noted that Lalko and Api (2006) evaluated essential oils and included analytical data on the composition of the oils as well as LLNA data on the identified major constituents and that these data should have been included in the evaluation and not just mentioned as other available scientific reports.

    - These data are included in the ICCVAM final Addendum for the LLNA applicability domain (see **Appendix D**).

The same commenter also agreed with the January 2008 ICCVAM draft recommendation that the LLNA is useful for the testing of metal compounds but questioned the importance or need to assess the LLNA's ability to detect metal allergens since the allergenic potential in humans of most known metals has already been established. Further, whether or not the LLNA is useful for testing nickel

compounds is of limited importance as nickel is a known human contact allergen. In addition, since only one of the 14 metal compounds with LLNA and human data was tested in an aqueous vehicle, the comparison did not add much value to the assessment, especially in light of the fact that the performance of the LLNA using aqueous vehicles was being assessed in this same report.

- ICCVAM considers it important to characterize the ability of the LLNA to appropriately detect the sensitization status of metals because metals may be components of formulated products that require testing to determine their skin sensitization potential.

The commenter also agreed with the January 2008 ICCVAM draft recommendation that an assessment of the suitability of the LLNA for testing substances in aqueous solutions should not be conducted until a sufficient quantity of quality data become available.

Two oral comments were relevant to the LLNA applicability domain.

1. One commenter noted that that the LLNA could be used to test pesticide formulations and supported the efforts of the EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations. If the LLNA is not accepted for testing formulations in the United States, international companies will be required to conduct both the LLNA and GP tests to satisfy the differing regulatory requirements for each formulation developed for global distribution. Such additional animal would be counter to the ICCVAM goal of reducing, refining, and replacing animal use in regulatory safety testing.

As outlined in the test method recommendations (see **Section 2.0**), ICCVAM recommends that the LLNA can be used for testing pesticide formulations, complex natural substances, dyes, metal compounds (except nickel), and substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle to maintain the test substance in contact with the skin (e.g., 1% Pluronic L92 [Boverhoff et al, 2008] so an adequate exposure is achieved, as demonstrated by a positive control response.

2. Another commenter expressed reservations about using the LLNA to test complex mixtures and formulations because it was developed to test single substances. The commenter also stated that, since most metals have already been tested (and their sensitization potential characterized), it does not seem worthwhile to try to optimize the LLNA for hazard and potency categorization for testing metals.

    - As outlined in the test method recommendations (see **Section 2.0**), the LLNA can be used for testing pesticide formulations, complex natural substances, dyes, metal compounds (except nickel), and substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle, to maintain the test substance in contact with the skin (e.g. 1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate exposure is achieved, as demonstrated by positive control results.
    - ICCVAM considers it important to characterize the ability of the LLNA to appropriately detect the sensitization status of metals because metals may be components of formulated products that require testing to determine their skin sensitization potential.

4.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. One public comment was received in response to this FR notice. The commenter made

a general comment that the members of SACATM do not represent a cross-section of the American public.

The SACATM charter indicates that the Committee shall consist of 15 members, including the Chair. Voting members shall be appointed by the Director, NIEHS, and include representatives from an academic institution, a State government agency, an international regulatory body, or any corporation developing or marketing new or revised or alternative test methodologies, including contract laboratories. Knowledgeable representatives from public health, environmental communities, or organizations using new or alternative test methodologies may be included as appropriate. There shall be at least one knowledgeable representative having a history of expertise, development, or evaluation of new or revised or alternative test methods from each of the following categories: (1) personal care, pharmaceutical, industrial chemicals, or agricultural industry; (2) any other industry that is regulated by one of the Federal agencies on ICCVAM; and (3) a national animal protection organization established under section 501(c)(3) of the Internal Revenue Code of 1986. The Director, NIEHS, shall select the Chair from among the appointed members of SACATM.

4.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. No public comments were received in response to this FR notice.

4.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method.

There were no public comments specific to the LLNA applicability domain.

Regarding the LLNA applicability domain, one SACATM member indicated that there was not enough data and information to offer an informed opinion.

As indicated in the January 2008 ICCVAM draft recommendations, more data and information were needed to make final recommendations for the LLNA applicability domain. NICEATM subsequently obtained additional data for pesticide formulations, dyes, and natural complex substances for inclusion in the updated draft Addendum that was evaluated by the Panel in April 2009.

4.7 Public Comments in Response to 74 FR 8974 (February 27, 2009): Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

NICEATM requested public comments on the updated drafts for the BRDs, Addendum, ICCVAM test method recommendations, and test method protocols for the second international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received three comments in response to this FR notice; one written comment, and two oral comments offered at the Panel meeting.

1. This was a general comment expressing concern that the extensive time and resources that ICCVAM has devoted to this evaluation has detracted from focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

ICCVAM considers the evaluations conducted to date have significant potential to further reduce and refine animal use, particularly where the use of the LLNA is precluded due to restrictions associated with the use of radioactivity. ICCVAM is also committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

The commenter further made one comment relevant to the LLNA applicability domain.

1. The commenter stated that the limited availability of data or the lack of clear definition of the test substance prevented a conclusive recommendation from the previous ICCVAM review for the use of the LLNA. The commenter noted that the updated recommendations from the current review of formulation and aqueous solutions offered a potential for expanded use, if overclassification was accepted (presumably by both the manufacturer and the regulatory agency). The commenter further noted that, in the interim, little had changed in the availability of comparative human data and they supported the ICCVAM recommendation that there is a need to identify relevant human data and human experience in order to continue to evaluate the applicability of LLNA to mixtures and aqueous solutions. The commenter indicated that this approach would provide the most valuable information and would not involve further animal testing, and therefore should be a priority.

    - ICCVAM will consider this comment when prioritizing future activities.

4.8 Public Comments in Response to 74 FR 19562 (April 29, 2009): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. No public comments were received in response to this FR notice.

4.9 Public Comments in Response to 74 FR 26242 (June 1, 2009): Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. One comment was received in response to this FR notice.

The commenter did not make a comment relevant to the LLNA applicability domain.

4.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009

The June 25-26, 2009, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method.

There were no public comments specific to the LLNA applicability domain.

In general, SACATM was supportive of the Panel report. However, there was general concern regarding the potential for over-labeling substances that may occur by using LLNA test results. They emphasized the need for developing non-animal test methods for identifying potential skin sensitizers.

Regarding the LLNA applicability domain, one SACATM member expressed concern about the limited additional data for the pesticide formulations. Compared to the original work on single substances, these data show that the pesticide formulations appear to produce false positives in the LLNA. The difference in sensitivity between the Buehler test and the GPMT was clarified. For the 22 substances for which there were comparative tests, 18 of the GPMTs were actually Buehler tests, so

there is a question as to whether they could have been concordant if they had been GPMTs. Strictly comparing the performance of the LLNA and the GPMT for those 22 substances, the accuracy is not great because the trend was to get a positive result more often in the LLNA.

As indicated in the ICCVAM final test method recommendations (**Section 2.1**), the potential for possible overclassification of pesticide formulations may be a limitation of the LLNA.

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